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14. ABSTRACT

Hantaviruses are negative-sense RNA enveloped viruses that are transmitted to humans in aerosols of rodent excreta. Hantaviral infections are associated with two significant human diseases: hemorrhagic fever with renal syndrome (HFRS) which is caused by "Old World" viruses found in Europe and Asia (Hantaan, Seoul, Puumala and Dobrava viruses) or hantavirus pulmonary syndrome (HPS) caused by the "New World" viruses of the Americas (Sin Nombre and Andes viruses). A goal of this project is to utilize high throughput genetic screens to define common cellular pathways, and broadly effective inhibitors targeting these pathways, that impact numerous hantaviruses. In the longer run, we hypothesize that the host factors identified by the proposed research will lead to new druggable targets for combating hantaviral infection.

15. SUBJECT TERMS
Hantavirus, Andes virus, Sin Nombre virus, Puumala virus, Dobrava virus, sterol regulatory complex, haploid genetic screen, cholesterol inhibitors,

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1. INTRODUCTION: Hantaviruses are negative-sense RNA enveloped viruses that are transmitted to humans in aerosols of rodent excreta. Hantaviral infections are associated with two significant human diseases: hemorrhagic fever with renal syndrome (HFRS) which is caused by viruses found in Europe and Asia (Hantaan, Seoul, Puumala and Dobrava viruses) or hantavirus pulmonary syndrome (HPS) caused by the "new world" viruses of the Americas (Sin Nombre and Andes viruses). A goal of this project is to utilize a powerful haploid cell genetic screening technology (3,4) to define common cellular pathways, and broadly effective inhibitors targeting these pathways, that impact numerous hantaviruses. In the longer run, we hypothesize that the host factors identified by the proposed research will lead to new druggable targets for combating hantaviral infection.
2. KEYWORDS: Hantavirus, Andes virus (ANDV), Sin Nombre virus (SNV), Puumala virus (PUUV), Dobrava virus (DOBV), Haploid screen, sterol regulatory complex (SRC), haploid genetic screen, cholesterol inhibitors, Major Task (MT).
3. ACCOMPLISHMENTS:

- What were the major goals of the project?
- The major goals of this project are as follows: 1) Analysis of the requirement for cholesterol synthesis by New and Old World hantavirus. 2) Identification and characterization of host factors involved in Old World Hantavirus entry. 3) Identification and characterization of host factors involved in New World Hantavirus entry. Each of these major goals was further divided into tasks and sub-tasks as detailed on the Statement of Work (attached).
- What was accomplished under these goals?

To date, the majority of the research outlined in the SOW has been completed for Specific Aims 1 and 2 (Major Tasks 1 3, 4 and 5). Accomplishments in the SOW are colored to denote those that are completed (green), in progress (blue) or pending (black). Specific details of the accomplished research are described in the following paragraphs and figures with the Specific Aim or Major Task that the work relates to indicated.

To identify host factors in addition to sterol regulatory complex (SRC) needed for efficient Andes virus infection (Specific Aim 1 and Major Task 3) we utilized genetically modified HAP1 cells. First, human haploid (HAP1) cells stably expressing the low density lipoprotein receptor (LDLR) were produced and characterized by flow cytometry using an antibody against LDLR. Specifically, the LDLR cDNA was inserted into the FCIV (FM5) lentiviral vector (generous gift of Dr. Jeffrey Milbrandt lab, Washington University). This vector uses the ubiquitin promoter to express the gene of interest and also expresses the Venus fluorescent protein via an internal ribosome entry site. Lentivirus (FCIV-LDLR) was produced by transient co-transfection of 293T cells with psPAX2 and pCMV-VSV-G then used to transduce HAP1 cells. Haploid, Venus+ cells were isolated by FACS sorting (Figure 1A). We had previously confirmed by flow cytometry of cell surface staining with an LDLR antibody that as expected LDLR is co-expressed in Venus+ cells. The smaller size of haploid cells by side scatter was employed as a gate along with Venus expression for cell sorting. The sorted cells were expanded and characterized by flow cytometry. In this analysis we found that the majority of the cells express Venus and display a Hoechst staining profile consistent with 59% 1n (haploid)

and 40% 2n (diploid or S phase haploid) cells (Figure 1B). Notably there is no 4n peak as would be expected for dividing diploid cells. Roughly 75 million HAP1-LDLR+ cells were then used to construct a insertionally mutagenized library using the LentiRET gene trap vector as described in the original proposal. Because these cells express LDLR they can efficiently scavenge cholesterol from the media and are not reliant upon cholesterol synthesis or the sterol regulatory complex. The mutagenized population was minimally expanded (2 doublings) and approximately 75 cells were selected by lethal infection with rVSV-ANDV as previously described (1). The remainder of the library was frozen for future use.

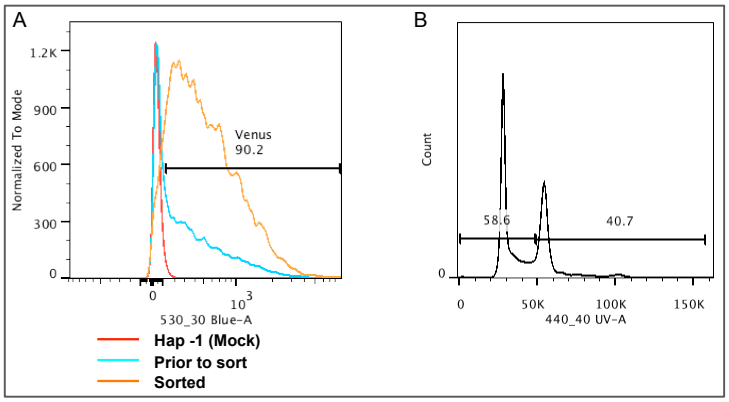


Figure 1. Generation of HAP1-LDLR+ cells. *HAP1 cells transduced with FCIV-LDLR were expanded and then 75 million cells were sorted for Venus expression and small size by side scatter. After expansion, the cells were characterized by flow cytometry for A) Venus expression and B) DNA content upon Hoechst staining.*

HAP1-LDLR+ cells surviving d10 post infection with rVSV-ANDV were collected and DNA was prepared for identification of the insertion sites (mutagenized genes). Using a PCR procedure that amplifies the LTR of the lentiviral mutagen and the flanking DNA, the insertion sites within the resistant cells were mapped onto the human genome and compared the insertion frequencies with those of our non-selected HAP1-LDLR+ library cells. To identify gene integrations enriched in the rVSV-ANDV surviving cells we compared integrations sites in this selected population to >300,000 unique integrations in the unselected library. The mapped integration sites corresponding to 30,011 unique reads and 6617 unique genes including 3864 genes with more than 1 unique integration. Table 1 lists the top hits that were identified in this screen after significance values were calculated and corrected for false discovery rate. The top hit is ATP6V0A1, a gene encoding a subunit of a vacuolar ATPase that is required to acidify endosomes. This finding validates the screen as an acidic endosome compartment is known to be essential for hantaviral infection. We have begun using siRNA knockdown to validate the hits from this screen. We have initially concentrated on Suppressor with Morphological effect on Genitalia 1 (SMG1) because the screen identified several SMG1 related genes (SMG1, SMG1P2 and SMG1P5). SMG1 is a phosphatidylinositol 3-kinase-related protein kinase (PIKK). Although no role has been previously described for SMG1 in viral infection, phosphatidylinositol kinases are known to be involved in endocytic uptake and trafficking prompting us to investigate SMG1 more fully.

Table 1. Hits from the HAP1-LDLR+ rVSV-ANDV screen.

GENE	p-value	# unique integrations
ATP6V0A1	0.0003	66
RHOA	0.0003	54
SMG1P2	0.0003	33
SMG1	0.0005	19
NOTCH2	0.0024	12
ANKRD36C	0.0025	25
LINC01128	0.0044	23
SMG1P5	0.0909	8

To investigate the potential role of the genes identified in this screen in hantaviral infection, two siRNA's specific for SMG1 were transfected into human U2OS cells then the cells were challenged with VSV-ANDV encoding GFP. Infection was measured by flow cytometry and normalized to the non-targeting siRNA. An siRNA targeting the vacuolar ATPase was employed as a positive control while a non-targeting siRNA was the negative control. Both siRNAs targeting SMG1 significantly impaired rVSV-ANDV infection yielding a 60-70% reduction while the positive control vATPase reduced infection by 80%. We are currently confirming knockdown of SMG1 at the RNA level by RT-qPCR and at the protein level by Western blot. In the coming months we will examine whether the SMG1 requirement is specific to ANDV or if other hantaviruses are similarly affected. Additionally, we will examine if more distantly related bunyaviruses or other virus families are affected by perturbation of SMG1. It will be important to verify that infection by the wild type hantaviruses, as opposed to the pseudotypes used for screening, is affected by SMG1. The potential role of SMG1 in hantavirtal entry is intriguing as protein kinases are very druggable targets. If the SMG1 kinase activity is required, then this could be an effective pharmacologic intervention for hantaviral infection.

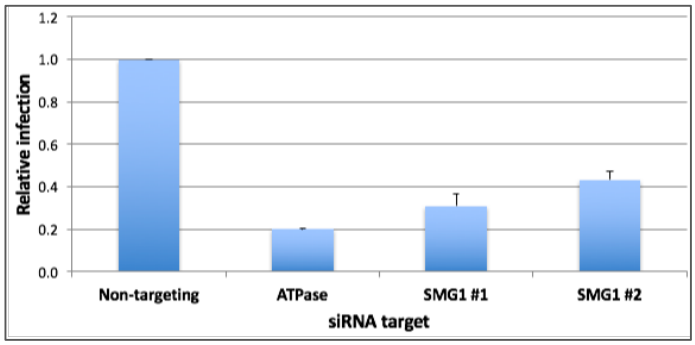


Figure 2. siRNAs targeting SMG1 inhibit Andes virus infection. *Human U2OS cells were transfected with siRNAs (Dharmacon) specific for SMG1 or the vacuolar ATPase. 48 hours after transfection the cells were infected with VSV carrying the Andes virus glycoproteins. 8 hours after infection the cells were fixed and analyzed for flow cytometry for the GFP marker encoded by VSV. Infection is normalized to control siRNA. Average of 2 independent experiments.*

For Specific Aim 2 of the proposal (Major Task 4) we performed a genome-wide loss-of-function genetic screen in haploid human cells utilizing a previously described library of insertionally

mutagenized human haploid cells (1). Viral challenge of these cells creates a genetic bottleneck, whereby cells harboring mutations in pro-viral genes undergo positive selection. Approximately 75 million mutagenized HAP1 cells were infected with a cytopathic, replication competent recombinant Vesicular Stomatitis Virus expressing the glycoproteins of the Old World hantavirus, Puumala (rVSV-PUUV). Several hundred colonies were observed 7-10 days after infection of mutagenized cells, while a parallel selection of non-mutagenized HAP1 cells yielded no survivors. We have previously observed a lack of survivors after challenging mutagenized HAP1 cells with a replication competent recombinant VSV bearing its endogenous glycoprotein (rVSV-VSV). Thus, the mutagenized HAP1 cells that survived rVSV-PUUV challenge likely carry mutations that confer specific resistance to infection mediated by the PUUV glycoprotein. Surviving cells were collected at day 10 and DNA prepared for identification of the insertion sites (mutagenized genes). Using a PCR procedure that amplifies the LTR of the lentiviral mutagen and the flanking DNA, the insertion sites within the resistant cells were mapped onto the human genome and compared the insertion frequencies with those of our non-selected library cells. 388,743 integration sites were mapped corresponding to 1229 unique reads and 726 unique genes. To identify gene integrations enriched in the VSV-PUUV surviving cells we compared integrations sites in this selected population to >1.5 million unique integrations in the unselected library. Significance values were calculated and corrected for false discovery rate. Analysis showed that, of those genes significantly enriched in integrations in the selected cells, four of the top five genes belong to the cholesterol regulatory pathway (Figure 3). These genes, Sterol Regulatory Element Binding transcription Factor 2 (SREBF2), Sterol Regulatory Element Binding transcription Factor Cleavage Activating Protein (SCAP), Site 1 Protease (S1P), and Site 2 Protease (S2P), are involved in the sensing and homeostasis of intracellular cholesterol, and had previously been identified in HAP1 genetic screens of factors needed for infection by the Old World hantavirus Andes (1). The p-values for the enrichment of these genes ranged from 2.3x10-5 (S2P) to 2.7x10-41 (SREBF2), supporting the conclusion that their disruption in our selected cells was not due to chance.

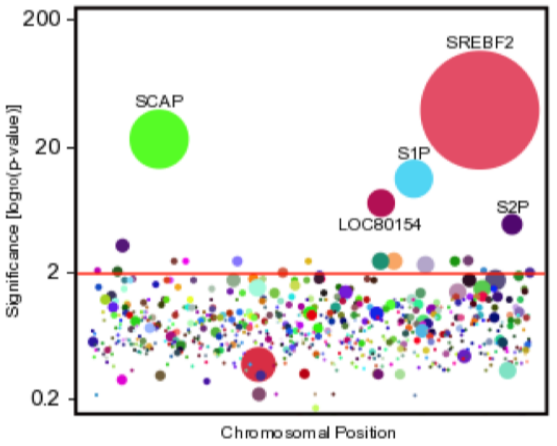


Figure 3. Haploid genetic screen identifies Puumala virus entry factors. *The frequency of inactivating integrations in genes in the selected population was compared to that in unselected cells. Statistical Enrichment Analysis was carried out using the Chi-Square Exact Test with false discovery rate correction. In this plot each dot represents a unique gene identified from integration site mapping in the HAP1-PUUV^R population. The size of the dot reflects the number of unique integration sites found within that particular gene. The y-axis denotes the significance values while the x-axis is position in the human chromosome.*

Having identified four members (SREBF2, SCAP, S1P, and S2P) of the cholesterol regulatory pathway in our genetic screen, we next sought to determine the importance of the genes for rVSV-

PUUV infection. Instead of using CRISPR mutatgenized cells as originally proposed, we instead utilized a set of well characterized Chinese Hamster Ovary (CHO) cell lines that are individually null for S1P, S2P, or SCAP. These cells are routinely utilized by the cholesterol synthesis field and thus results using these cell lines are readily comparable to that of others in this field. We challenged the CHO knockout cell lines as well as parental CHO with rVSV-PUUV, rVSV-ANDV, or rVSV-VSV. As expected, rVSV-VSV infection was at most only moderately inhibited, showing approximately 70% less infection in SCAP null compared to parental cells (Figure 4). The effect of pathway disruption upon rVSV-PUUV infection was far more robust, with at least 95% less infection in all null cell types relative to parental. This result is similar to that observed for the New World hantavirus, Andes (ANDV) (1). Our results using these mutant cells further support the cholesterol regulatory complex as important for rVSV-PUUV infection, showing a robust dependence of the virus upon all observed genes.

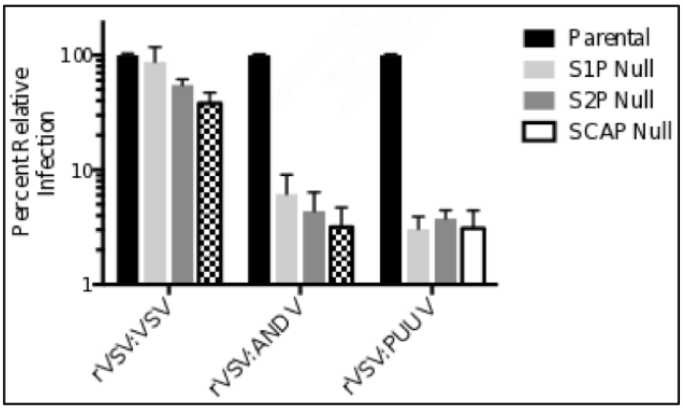


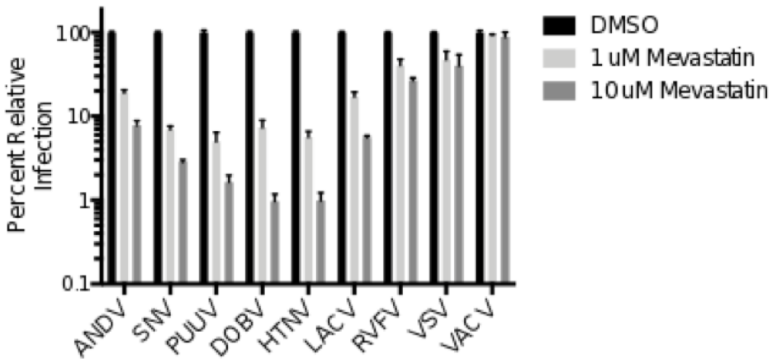
Figure 4. Genetic disruption of the sterol regulatory complex inhibits hantaviral entry. *CHO cells null for SCAP, S1P, or S2P were infected with recombinant VSV bearing the glycoproteins of PUUV, ANDV, or VSV. Infection was quantified by flow cytometry and normalized to infection of wild-type cells (CHO-K1). N=6 Bars=SEM.*

Overall, the genes identified as critical for infection by viruses carrying the glycoproteins of an Old World hantavirus, Puumala, are nearly identical to those found using the New World hantavirus Andes virus that we previously described (1). These results extend our previous work by demonstrating that all hantaviruses have a profound requirement for cholesterol synthesis and suggest that perturbation of cholesterol levels may prove effective at preventing hantaviral infection in vivo. This requirement for cholesterol synthesis by prototypic New and Old World hantaviruses is further demonstrated in Figure 5 using mevastatin, an FDA-approved pharmacologic inhibitor of cholesterol synthesis in cultured cells. VSV carrying its own glycoprotein is used as a control. Figure 5 clearly demonstrates that New World hantaviruses, Andes (ANDV) and Sin Nombre (SNV) as well as the Old World hantaviruses (Puumala; PUUV, Dobrava; DOBV, Hantaan; HTNV) are impaired similarly to ANDV and PUUV by mevastatin. In contrast the more distantly related bunyaviruses Rift Valley fever virus (RFTV) and LaCrosse virus (LACV) are minimally impacted by this inhibitor while the controls VSV-G and vaccinia show no effect.

Overall, our analysis has clearly demonstrated a role for cholesterol biosynthetic pathway in infection mediated New and Old World hantaviral glycoproteins. To address the mechanism of the cholesterol requirement for hantaviral infection (Major tasks 2 and 6) we have used a series of inhibitors of the cholesterol synthesis pathway to determine if there is a direct requirement for

cholesterol or another sterol or by-product. To address this question two inhibitors of steps late in the cholesterol synthesis pathway were examined for their effect upon VSV-PUUV infection. We find that perturbation of late steps in cholesterol synthesis dramatically reduces PUUV infection (data not shown). Additionally, entry of the New World hantavirus Andes is also significantly impaired by these compounds.

Figure 5. The FDA-approved cholesterol synthesis inhibitor Mevastatin inhibits infection by prototypic New & Old World hantaviruses. *Two concentration of mevastatin were used to treat A549 cells for 12 hours prior to infection with a GFP-expressing VSV vector pseudotyped with the viral glycoproteins from either New World or Old World hantaviruses. VSV and Vaccinia viruses were used as controls.*



Based upon these results, we hypothesized that cholesterol itself might be required for efficient endosomal transport of incoming virus or fusion of the viral and host membranes. We have previously reported a defect in internalization of Andes virus when cholesterol synthesis is pharmacologically inhibited (Petersen et al 2104). To further support our findings and expand our understanding of hantaviral dependence upon cellular cholesterol for optimal infection, we utilized the sterol-binding agent methyl-β-cyclodextrin (MBCD). Depending on the relative ratios of free and MBCD-bound cholesterol, MBCD is able to extract from or add to the cholesterol content of membranes. Because of this, the direct effects of cholesterol depletion and reconstitution upon viral infection can be readily observed.

Human A549 cells were treated with MBCD or vehicle in delipidated media, followed by rinsing and a 45-minute incubation with delipidated media containing either vehicle alone or MBCD loaded with cholesterol. After treatment, cells were infected with rVSV-PUUV, rVSV-ANDV, or rVSV-VSV in delipidated media. Cholesterol depletion had only a moderate impact on rVSV-VSV infection, decreasing it by approximately 50% (Fig. 6A). A more robust inhibition of infection was seen for both rVSV-PUUV and rVSV-ANDV, with infection of these viruses decreased by greater than 80% and 94%, respectively. Importantly, infection of all three viruses was rescued by cholesterol reconstitution, supporting the specific role of cholesterol in the phenotype.

We next sought to illuminate the step of the viral lifecycle involved in the phenotype. As mentioned we have previously shown that pharmacologic or genetic cholesterol depletion impedes hantaviral endocytosis. However, considering the importance of cholesterol in membrane content, organization, and fluidity, we hypothesized that cholesterol depletion might be acting at several steps during hantaviral entry and thus chose to directly investigate the impact of cholesterol depletion upon hantaviral membrane fusion with the host membrane.

To do so, we again utilized MBCD treatment and observed the ability of recombinant viruses to fuse with the cellular membrane at low pH following cholesterol depletion or supplementation. It is well established that hantaviruses require a low pH environment to trigger virus-host membrane fusion and we mimicked this using a brief low pH treatment. Human A549 cells were incubated with delipidated media containing vehicle, MBCD, or MBCD loaded with cholesterol. Cells were subsequently rinsed and rVSV-PUUV, rVSV-ANDV, or rVSV-VSV was added in cold delipidated media and allowed to bind before cells were rinsed and 37 C media adjusted to pH 4.9 was added for 90 seconds. Following acid bypass, we incubated the cells in delipidated media containing NH_4Cl to limit infections to those initiated by low pH fusion at the cell membrane.

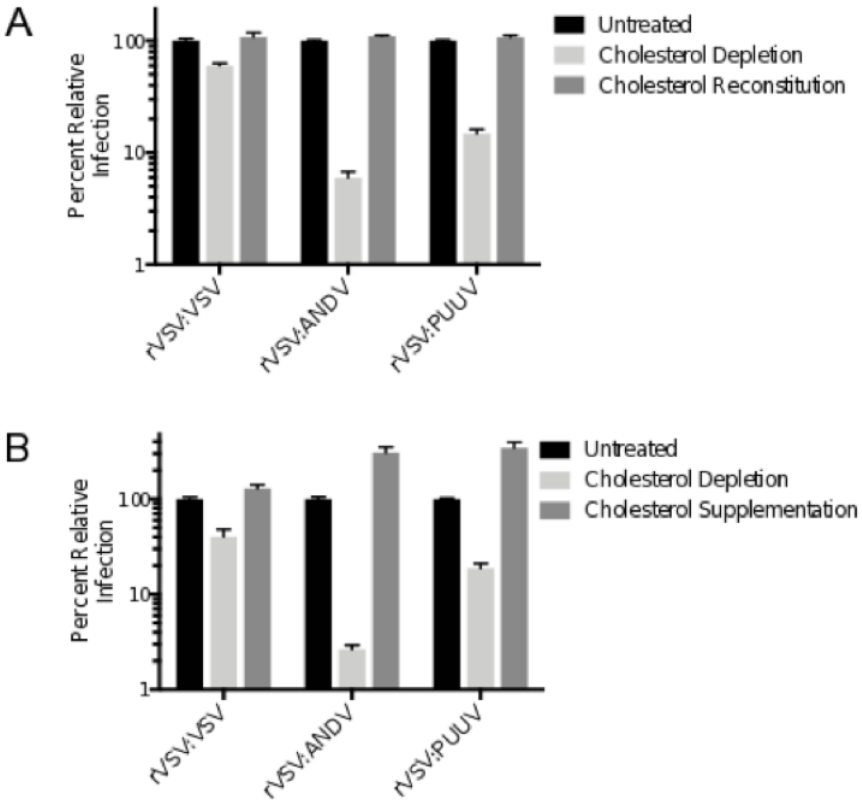


Figure 6. *Hantaviral infection requires cholesterol for optimal membrane fusion.* ***(A)*** *A549 cells were treated with vehicle or MBCD or deplete plasma membrane cholesterol. Depleted cells were further treated with vehicle or cholesterol-loaded MBCD to reconstitute cellular cholesterol before all cells were infected with rVSV-PUUV, rVSV-ANDV, or rVSV-VSV. Infection was quantified by flow cytometry and normalized to vehicle controls. N=6 Bars=SEM.* ***(B)*** *A549 cells were either depleted of membrane cholesterol with treatment with MBCD, had their cholesterol content supplemented by incubation with cholesterol-loaded MBCD, or treated with vehicle. Acid bypass of the VSV pseudotypes was then carried out at pH 4.9, followed by incubation in NH_4Cl-containing media and quantification of infection by flow cytometry. Infection was normalized to vehicle controls. N=6 Bars=SEM.*

The results of infection by acid-bypass following cholesterol depletion or supplementation closely parallels the previous MBCD experiment. rVSV-VSV showed a moderate decrease in fusion following cholesterol depletion, with slightly greater than 60% inhibition (Figure 6B). PUUV infection was decreased approximately 80% in fusion following cholesterol depletion, while rVSV-ANDV showed more than 97% decreased fusion. The greater cholesterol dependence of the hantaviruses compared to rVSV-VSV was also observed in their response to cholesterol supplementation, where we observed both rVSV-PUUV and rVSV-ANDV fusion increase by approximately three fold compared to untreated cells. Together, our results utilizing MBCD to alter cellular cholesterol levels support our earlier drug and genetic results, showing a significant dependence of the featured hantaviruses upon cellular cholesterol for infection. The acid bypass data identifies a major site of this

dependence, supporting a block to virus fusion with the host membrane to be a key point of the observed inhibition. Coupled with our prior observation showing impaired trafficking (1), this suggests that cholesterol may work at several points during hantaviral infection.

We are also continuing our studies developing a protein system that will allow microscopic tracking of incoming viral particles (Major tasks 2, 6 and 9) to determine the point at which cholesterol is required for hantaviral infection by employing an epitope tagged protein that is co-incorporated along with the hantaviral glycoproteins into the VSV pseudotypes for microscopic analysis of viral entry. Originally, we proposed using a simple protein consisting of a membrane anchor (the VSV-G stem region) with an appended HA epitope tag on the extracellular surface (HA-Gstem). We modified this plan to include a newly described small peroxidase protein, APEX (2,3), in addition to Gstem and an epitope tag. In addition, 2 other constructs with differing membrane anchoring units were developed with a goal of identifying the one that is most effectively incorporated into virions. We have called these proteins Virion-associated APEX (VaAPEX). The 3 constructs being evaluated are VaAPEX-G, (VSV transmembrane anchor), VaAPEX-TM (Tva membrane anchor) and VaAPEX-GPI (glycolipid membrane anchor).

The APEX technology (2, 3) is based upon the generation of short-lived free radicals that covalently bind electron-rich amino acids in close proximity (~20nm) to the APEX enzyme and can therefore theoretically be used to label the area of the cell or cellular compartment in which the incoming virus localizes. Substrates for fluorescent microscopy (as proposed in the original grant) or dense labels for use in electron microscopic analysis are available. The ability to utilize EM or fluorescence microscopy makes this a much more flexible system to determine the point at which the cholesterol pathway is needed in hantaviral infection. To date we have produced three variants of the APEX tagging constructs and have evaluated their cell surface expression (required for incorporation into VSV) by both flow cytometry and microscopy and the tested functionality of the APEX peroxide on the cell surface. All three are well expressed at the cell surface and functional as determined by biotin-labeling of the cell surface (Figure 7). Single channel analysis of the fluorescently stained HeLa cells expressing the VaAPEX constructs demonstrates intense GFP signal indicating very efficient surface labeling. Indeed, in Figure 7 where both channels are displayed the red flourescence for the epitope tag is not easily seen because of the intense GFP (biotin) signal.

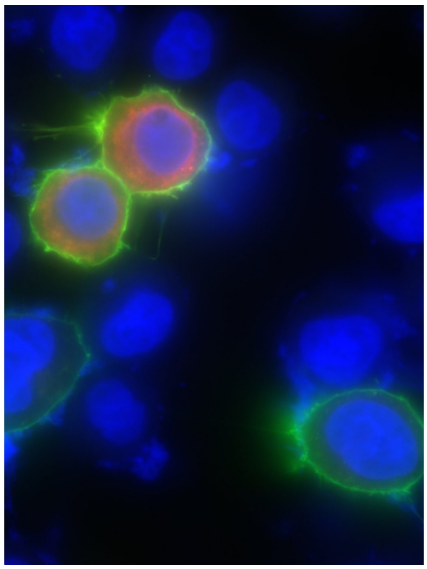


Figure 7. Function of the VaAPEX system in HeLa cells. *HeLa cells were transfected with 400 ng plasmid expressing VaAPEX-TM then labeled and fixed 26 hours post transfection. Biotin-phenol was used to label cell surface proteins in cell that express VaAPEX-TM. Cells were then permeablilized and stained for biotin (green) or the FLAG epitope tag (red) on VaAPEX.*

VaAPEX-G, VaAPEX-TM and VaAPEX-GPI were evaluated for their ability to incorporate into pseudotyped particles (MT2, subtasks 1 &

2) by western blot of released virions and also single particle analysis by microscopy (data not shown). All three are effectively incorporated into VSV, HIV or MLV based viral particles along with hantaviral glycoproteins (MT2, subtask 2). We find that transient expression gives much greater incorporation that stable cell lines and will therefore not generate stable Vero lines as was proposed. Moreover, using biotin labeling of the virions themselves we have found that APEX is enzymatically active in the released viral particles (data not shown). Thus, we have in place and have optimized (MT2, subtask 3) a system to follow incoming viral particles in cells where cholesterol homeostasis or SMG1 are impaired.

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6.

- What opportunities for training and professional development has the project provided?
- "Nothing to Report."
- How were the results disseminated to communities of interest?
- Results of the initial experiments analyzing Puumala virus were presented by Ben Dyer in July at the 2015 American Society for Virology Meeting in Ontario, Canada.

- The APEX technology for proximity labeling of viral interacting proteins was presented by Ben Dyer in July at the 2016 American Society for Virology Meeting at Virginia Tech University.
- What do you plan to do during the next reporting period to accomplish the goals?
- In the short term we will further analyze the requirement for SMG1 in hantaviral infection using additional siRNA experiments, general PIKK inhibitors and specific SMG1 inhibitors if they become available. We have completed characterization of the APEX proteins and are ready to initiate microscopy experiments to determine how these inhibitors of entry work (Major Tasks 2 & 6). Finally, we are submitting a manuscript describing the Puumala virus experiments.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

- What was the impact on the development of the principal discipline(s) of the project?
- These are the first forward genetic experiments designed to elucidate cellular factors needed for hantaviral entry into host cells. Thus the knowledge gained here will not only inform the basic biology of hantaviral replication but should also describe potential inhibitors of hantaviral infection. To date we have found cholesterol synthesis inhibitors are broadly applicable against New and Old World hantaviruses. Recent results hint that protein kinase (specifically PIKK) inhibitors may also be useful for combating hantaviral infection. Additionally, we have developed replication-competent recombinant viruses that carry the hantaviral glycoproteins but are safer and can therefore be handled in lower biosafety levels. Although we developed them to facilitate genetic screening, these viruses should be of utility to other groups developing hantaviral infection inhibitors and especially vaccines as a similar VSV construct forms the basis for an effective Ebola vaccine.
- What was the impact on other disciplines?
- "Nothing to Report."
- What was the impact on technology transfer?
- "Nothing to Report."
- What was the impact on society beyond science and technology?
- "Nothing to Report."

5. CHANGES/PROBLEMS: As described in the report, a cryogenic storage system failure has modestly delayed our progress. We have already begun re-generating the lost libraries and expect to “catch-up” this year.

- Changes in approach and reasons for change
- A very minor change is that we are utilizing a new technology (APEX) that allows us to more effectively perform the studies on hantaviral entry and the mechanism of various inhibitors. Overall this change is of minimal impact on the design of the

proposed experiments. Additionally, based on the results presented, we have recently identified SMG1 as a new potential host factor needed for VSV-ANDV infections. We plan to spend significant effort to evaluate this lead because of the druggable nature of the target and possible use for therapeutic intervention in hantaviral infection.

- Actual or anticipated problems or delays and actions or plans to resolve them
- A June 2015 a cryogenic storage unit failure caused the loss of a large HAP1 lentiviral mutagenized library and also a LDLR expressing HAP1 library. This delayed experiments described in Major Task 3 and Major Task 7. Using backup HAP1 cells from a separate cryogenic storage facility we were able to re-construct these reagents however we were delayed by several months. We anticipate no further delays from this storage failure.
- Changes that had a significant impact on expenditures
- Nothing to Report
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
- Nothing to Report
- Significant changes in use or care of human subjects
- Nothing to Report
- Significant changes in use or care of vertebrate animals.
- Nothing to Report
- Significant changes in use of biohazards and/or select agents
- Nothing to Report

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- Publications, conference papers, and presentations
- Journal publications. NA
- Books or other non-periodical, one-time publications. NA
- Other publications, conference papers, and presentations.
- Presentation entitled Discovery of "Developing a novel proximity-based assay for discovering host-virus interactions" by Ben Dyer (student) at the 35th Annual Meeting of the American Society for Virology. July 2016.
- Website(s) or other Internet site(s) NA
- Technologies or techniques We have generated the technology to produce lentiviral mutagenized HAP1 libraries that can be used to identify important genes for numerous cellular processes. This technology will be shared freely with the scientific community.
- Inventions, patent applications, and/or licenses NA
- Other Products We have generated recombinant VSV viruses that carry various hantaviral glycoproteins. These viruses will be of general utility to groups analyzing hantaviral entry and/or inhibitors of hantaviral infection including antibodies and vaccines.

Additionally, we have created cell lines in which genes encoding SRC factors have been inactivated by CRISPR technology which will be of utility to others who work in this area. These reagents will be shared freely with the scientific community

- PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
- What individuals have worked on the project?

▪ Name:	Paul Bates
▪ Project Role:	PI
▪ Researcher Identifier (e.g. ORCID ID):	PFBATES (eRA Commons ID)
▪ Nearest person month worked:	12
▪ Contribution to Project:	Directed Project, coordinated team, analyzed data
Funding Support:	NIH R01AI081913, this award
▪ Name:	MJ Drake
▪ Project Role:	Graduate Student
▪ Researcher Identifier:	MJDRAKE (eRA Commons ID)
▪ Nearest person month worked:	9
▪ Contribution to Project:	Performed experiments, analyzed data
Funding Support:	NIH T32AI007324, this award
Name:	Natalia Shalginskikh
Project Role:	Postdoctoral Fellow
Researcher Identifier:	
Nearest person month worked:	12
Contribution to Project:	Performed experiments, analyzed data
Funding Support:	This award
Name:	Ben Dyer
Project Role:	Graduate Student
Researcher Identifier:	
Nearest person month worked:	12
Contribution to Project:	Performed experiments, analyzed data
Funding Support:	This award, NSF Fellowship

Name:	Steven Bart
Project Role:	Graduate Student
Researcher Identifier:	SMBART (eRA Commons ID)
Nearest person month worked:	6
Contribution to Project:	Performed experiments, analyzed data
Funding Support:	This award, NIH T32AI007324

-
- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
- A NIH grant (1R21 AI 129531-01) entitled "Defining Zika virus-host cell interactions using forward genetic screens" with Dr. Bates as PI received a 19 impact score and was funded.
- What other organizations were involved as partners?
- Nothing to Report.

7. SPECIAL REPORTING REQUIREMENTS
- COLLABORATIVE AWARDS:
- Nothing to Report.
- QUAD CHARTS (revised): Attached
-

APPENDICES: SOW (revised) - Attached

Discovery of Host Factors and Pathways Utilized in Hantaviral Infection

Log Number: PR130590

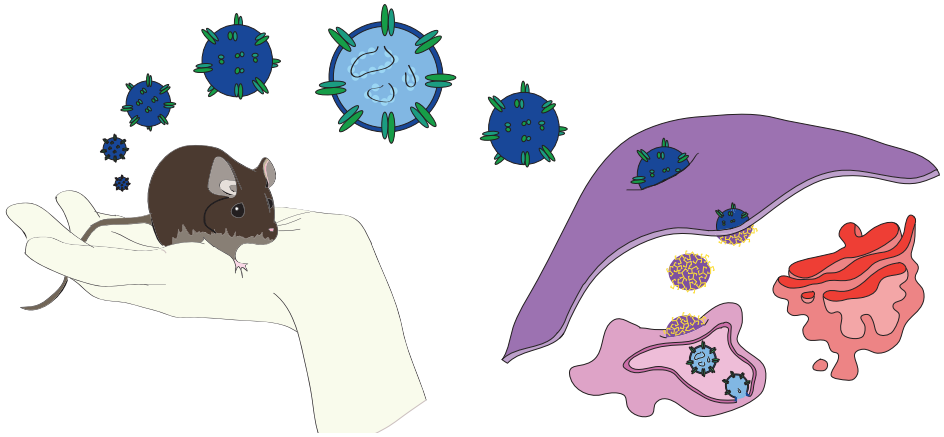
PI: Paul Bates, PhD **Org:** University of Pennsylvania **Award Amount:** $1,183,764

Study Aims

- Analysis of the requirement for cholesterol synthesis by New and Old World hantavirus
- Identification and characterization of host factors involved in Old World Hantavirus entry
- Identification and characterization of host factors involved in New World Hantavirus entry

Approach

The aim of this research is to identify key human factors exploited by hantaviruses viruses during entry into a host cell in order to gain basic knowledge regarding hantavirus infection and then to utilize this information for development of preventative or therapeutic medical treatments. The approach to be used employs cutting edge genetic screening, genome engineering and DNA sequencing technologies to address this important topic.



This drawing depicts the process of hantavirus infection starting with excretion from the rodent host, aerosol transfer , then contact with a human cell membrane (purple), endocytic uptake and finally penetration into the host cell.

Timeline and Cost

Major Activities CY	14	15	16
Characterize New & Old World hantavirus infection in sterol regulatory complex (SRC) knockout cell lines.	✓		
Identify and characterize host factors involved in Old World Hantavirus entry		✓	
Identify and characterize host factors involved in New World Hantavirus entry			
Estimated Budget (Total Cost $K)	396	396	396

Updated: (November 8, 2016)

Goals/Milestones

CY14 Goals – Characterize New & Old World hantavirus infection in sterol regulatory complex (SRC) knockout cell lines. ✓

- Identify host factors in addition to sterol regulatory complex needed for efficient Andes virus infection.
- Analyze the point at which the SRC impacts ANDV infection.

CY15 Goal –Identification and characterization of host factors involved in Old World Hantavirus entry.

- Screening of mutant cell library for Puumala and Hantaan virus entry factors. ✓
- Confirm candidate genes. ✓
- Characterize mechanism of inhibition.

CY16 Goal – Identification and characterization of host factors involved in New World Hantavirus entry.

- Generate recombinant Sin Nombre-VSV virus & stocks.
- Screen mutant cell library for Sin Nombre virus entry factors.

STATEMENT OF WORK – *revised* November 8, 2016

Site: University of Pennsylvania
Department of Microbiology
Philadelphia, PA
PI: Paul Bates

Green indicates completed or nearly complete task, orange indicates modified task, blue indicates task in progress, red denotes task that is behind schedule & indicates revised timeline

Overall Goal: *The overall goal is to use cutting edge genetic screens of human haploid cells and high throughput sequencing methods to identify cellular factors or pathways needed for hantavirus infection then to explore inhibitors (genetic and pharmacologic) of these host factors as potential antivirals.*

Specific Aim 1: Analysis of the requirement for cholesterol synthesis by New and Old World hantavirus	**months**	
Major Task 1: Characterize New & Old World hantavirus infection in sterol regulatory complex (SRC) knockout cell lines		
Subtask 1: Complete a panel of VSVs representative of Old (PUUV, HTNV, DOBV, SEOV) & New World (ANDV, SNV) hantaviruses	1-4	B. Dyer
Subtask 2: Create CRISPR knockout cell lines for SCAP, S1P and S2P in HAP1 and A549 (epithelial) cells	1-3	MJ Drake
Subtask 3: Quantify infection of New and Old World hantavirus rVSVs in cells where SRC function is impaired by CRISPR mediated gene disruption, siRNA (SCAP, SREBF2, S1P, S2P, HMG-CoA) or inhibitors (PF-429242, Mevastatin)	4-6	MJ Drake & B. Dyer
Subtask 4: Analyze synergy of statins and other SRC inhibitors	7-10	B. Dyer
Milestone(s) Achieved: Determination of SRC requirement for New & Old World hantaviral infections; publication of 1 peer reviewed paper	14	P. Bates, MJ Drake & B. Dyer
Major Task 2: Analyze the point at which the SRC impacts ANDV infection.		
Subtask 1: Produce HAstem expressing Vero cell line – sort for high expression by FACS Construct APEXstem (alternative tracking system)	15	B. Dyer
Subtask 2: Produce rVSV-ANDV or VSV-ANDV pseudotypes with incorporated HAstem (VaAPEX)	16	B. Dyer
Subtask 3: Optimize assays analyzing early hantaviral entry steps	17-18	B. Dyer
Subtask 3a: Analyze the effect of cholesterol depletion/reconstitution with MBCD on PUUV infection (added experiments from original SOW)		B. Dyer
Subtask 4: Analyze entry steps in SRC KO or inhibitor treated cells using rVSVs	19-23	B. Dyer
Subtask 5: Confirm findings of subtask 4 with bona fide ANDV	24-26	B. Dyer
Milestone(s) Achieved: Elucidation of the mechanism of cholesterol requirement of ANDV host cell entry; publication of 1 peer reviewed paper	28	P. Bates & B. Dyer
Major Task 3: Identify host factors in addition to sterol regulatory complex needed for efficient Andes virus infection	(Revised timeline due to	

	loss of library)	
Subtask 1: Produce LDLR expressing HAP1 library	1-5 (12-18)	N. Shalginskikh
Subtask 2: Screen LDLR expressing HAP1 library with rVSV-ANDV to identify additional host entry factors	6-8 (18-20)	N. Shalginskikh
Subtask 3: Identify integration sites in LDLR expressing ANDV-resistant cells	9-10 (20-24)	N. Shalginskikh & F. Male
Subtask 4: Confirm candidate genes using CRISPR knockout cells, RNAi and/or shRNA	11-16 (24-26)	N. Shalginskikh & B Dyer
Milestone(s) Achieved: Identification of genes needed for efficient ANDV infection in cells with high cholesterol levels; publication of 1 manuscript	26-28	N. Shalginskikh & P. Bates
Specific Aim 2: Identification and characterization of host factors involved in Old World Hantavirus entry		
Major Task 4: Screening of mutant cell library for Puumala and Hantaan virus entry factors		
Subtask 1: Generate master stocks of rVSV-PUUV for library screening.	1-4	B. Dyer
Subtask 2: Screen HAP1 LentiET mutagenized library with rVSV-PUUV. Collect cells surviving virus challenge.	4-8	B. Dyer
Subtask 3: Use LM-PCR to identify integration sites in starting HAP1 library	9-12	B. Dyer , MJ Drake & F. Male
Subtask 4: Use LM-PCR to identify integration sites in rVSV-PUUV selected cells	9-12	B. Dyer , MJ Drake & F. Male
Milestone(s) Achieved: identification of candidate genes needed for Old World hantavirus entry	13	B. Dyer , MJ Drake & P.Bates
Major Task 5: Confirm candidate genes using VSV pseudotype and parental hantavirus infection of CRISPR knockout, RNAi, shRNA cells and/or pharmacologic inhibitors.		
Subtask 1: Create knockout cell lines for PUUV hits using CRISPR technology (used well characterized CHO knockout cells instead)	13-17	B. Dyer
Subtask 2: Quantify infection by rVSV-HTNV and rVSV-PUUV in KO cell lines (CHO cells null for SP1, SP2 and SCAP)	17-20	B. Dyer , MJ Drake
Subtask 3: Identify and obtain pharmacologic or genetic inhibitors of candidate genes	13-14	B. Dyer , MJ Drake
Subtask 4: Quantify infectivity of rVSV-HTNV and rVSV-PUUV in presence of pharmacologic or genetic inhibitors (siRNAs)	17-20	B. Dyer , MJ Drake
Subtask 5: Quantify infectivity of parental HTNV & PUUV in KO cells & in presence of pharmacologic or genetic inhibitors	21-25	B. Dyer , MJ Drake

Major Task 6: Characterize mechanism of inhibition by identified genes and inhibitors		
Subtask 1: Produce and purify rVSV-PUUV(APEXstem) & rVSV-HTNV(APEXstem)	25	S. Bart & B.Dyer
Subtask 2: Optimize high content assay analyzing early entry steps	26-27	S. Bart & B.Dyer
Subtask 3: Analyze entry steps in KO or inhibitor treated cells with high content analysis using rVSV-PUUV(APEXstem) & rVSV-HTNV(APEXstem)	27-31	S. Bart & B.Dyer
Subtask 3a: Analyze the effect of cholesterol depletion/reconstitution with MBCD on PUUV infection (added experiments)		B. Dyer
Subtask 4: Confirm findings of subtask 3 with parental PUUV & HTNV	32-34	S. Bart
Milestone(s) Achieved: Identification of genes needed for efficient Old World hantavirus infection; publication of 1-2 peer reviewed papers	36	B. Dyer, MJ Drake, & P. Bates
Specific Aim 3: Identification and characterization of host factors involved in New World Hantavirus entry		
Major Task 7: Screening of mutant cell library for Sin Nombre virus entry factors		
Subtask 1: Generate master stocks of rVSV-SNV for library screening	17	B. Dyer
Subtask 2: Produce new HAP1 Lenti library. (replace library lost in storage failure). Screen HAP1 LentiET mutagenized library with rVSV-SNV. Collect cells surviving virus challenge.	17-18	B. Dyer & N. Shalginskikh
Subtask 3: Use LM-PCR to identify integration sites in virus-resistant cells & parental library	19-20	N. Shalginskikh & F. Male
Subtask 4: Identify list of hits – genes or pathways significantly enriched in selected cell population	21	N. Shalginskikh & F. Male
Milestone(s) Achieved: identification of candidate genes needed for New World hantavirus entry	21	
Major Task 8: Confirm candidate genes using VSV(SNV) and SNV infection of CRISPR knockout, RNAi and/or shRNA cells.		
Subtask 1: Create knockout cell lines using CRISPR technology	22-23	B. Dyer & S. Bart
Subtask 2: Quantify infectivity of rVSV-SNV and SNV in knockout cells	23-24	B. Dyer & S. Bart
Subtask 3: Identify and obtain pharmacologic or genetic inhibitors of candidate genes	24	B. Dyer & S. Bart
Subtask 4: Quantify infectivity of rVSV-SNV and SNV in presence of pharmacologic or genetic inhibitors	25-27	B. Dyer & S. Bart
Subtask 5: Cross screen other hantaviruses for effect of confirmed genes and	27-28	B. Dyer & S.

inhibitors		Bart
Milestone(s) Achieved: Identification of genes needed for efficient SNV infection; publication of 1 peer reviewed paper	31	B. Dyer, S. Bart, P. Bates
Major Task 9: Characterize mechanism of inhibition of SNV by identified genes and inhibitors		
Subtask 1: Produce and purify rVSV-SNV(HAstem)	29	B. Dyer
Subtask 2: Optimize high content assay analyzing early SNV entry steps	30-31	B. Dyer
Subtask 3: Analyze entry steps in KO or inhibitor treated cells with high content analysis using rVSV-SNV(HAstem)	32-35	B. Dyer
Subtask 4: Confirm finding of subtask 3 with SNV	35-36	B. Dyer
Milestone(s) Achieved: Identification of genes needed for efficient SNV infection; publication of 1 peer reviewed paper	36	B. Dyer. S. Bart & P. Bates

Requested information:
- *The genotypes (where applicable) of mouse strains used* - Not Applicable
- *Where applicable, the approximate number of samples (both control and experimental)* – Not Applicable
- Abbreviations & Acronyms:

SRC sterol regulatory complex
VSV vesicular stomatitis virus
PUUV Puumala virus
HTNV Hantaan virus
DOBV Dobrava virus
SEOV Seoul Virus
ANDV Andes virus
SNV Sin Nombre virus
rVSV-ANDV recombinant VSV with ANDV glycoproteins
rVSV-HTNV recombinant VSV with HTNV glycoproteins
rVSV-PUUV recombinant VSV with PUUV glycoproteins
CRISPR clustered regularly interspaced short palindromic repeats
SCAP SREBP cleavage-activating protein
S1P site 1 protease
S2P site 2 protease
SREBF2 sterol regulatory element binding transcription factor 2
HMG-CoA 3-hydroxy-3-methyl-glutaryl-CoA reductase
HAstem Influenza Hemagglutinin stem region
rVSV-PUUV(HAstem) recombinant VSV with PUUV & HAstem glycoproteins
rVSV-SNV(HAstem) recombinant VSV with SNV & HAstem glycoproteins